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WITNESS my hand this
Second day of November 2000

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AUSTRALIA

Patents Act 1990

Unisearch Limited

PROVISIONAL SPECIFICATION

Invention Title:

Method and apparatus for culturing cells

The invention is described in the following statement:

Method and apparatus for culturing cells

The present invention relates to a method and apparatus for culturing cells. The invention is particularly concerned with a method and apparatus for growing and maintaining cells *in vitro* at high cell densities.

Cell culturing is important for cell biology and immunological studies and for use in medical therapies such as cell therapy. Particular examples of cell therapy include blood stem cell transplantation to regenerate blood production after high dose therapy for cancer; cellular immunotherapy to eliminate residual cancer cells or reconstitute immunity to viruses; and somatic gene therapy as a cure for genetic and viral diseases (e.g. Haemophilia, HIV).

Cell therapy generally requires large-scale production of cells. Flask or bag tissue culture techniques are relatively wasteful. Using these techniques, mammalian cells grow to a maximum density of 1-2x10^6 cells/ml. At this cell concentration, the media must be replenished because glucose depletion and lactate accumulation inhibit cellular metabolism. The process is also wasteful since proteins are discarded even though their levels are not depleted. The current cost of serum free media that is suitable for use in clinical trials is at least $A2000-$3000 per litre; the major proportion of the cost relates to manufacture of clinical grade human albumin, low density lipoproteins and recombinant growth factors. Thus for clinical applications which require transplants of up to 10^{10} cells, the cost of media alone will be prohibitively expensive.

There is a need for more cost-effective technologies for the generation of large numbers of cells. Applicant's US Patent No. 5,763,194, the disclosure of which is incorporated herein by reference, describes a cell separation device and method based on the use of a semi-permeable substrate in the form of an array of hollow fibres provided internally with a ligand reactive

with the desired cell type. US Patent No. 5,763,194 also discloses the use of the cell separation device for cell expansion.

We have carried out further research based on the hypothesis that cells will grow at high density in culture systems that maintain metabolites, such as glucose and lactate, within their physiological ranges and in which there is recycling or retention of protein components. Recycling or retention of protein components provides a high density culture system that is potentially more cost-effective.

In particular, the present inventors have found that by appropriate selection of the permeability of a semi-permeable substrate, cells captured and grown inside the semi-permeable substrate can be grown to concentrations up to 40-50 times higher than the concentration of cells that can be supported in conventional culture systems (T flasks or Teflon bags).

Accordingly, in a first aspect, the present invention provides a method for culturing one or more type(s) of cells, the method including:

providing a semi-permeable substrate having said cells on one side thereof, wherein said semi-permeable substrate is permeable to metabolites but is substantially impermeable to at least one protein required for proliferation, differentiation and/or genetic modification of said cells;

contacting said cells with a culture medium including at least one protein required for proliferation, differentiation and/or genetic modification of said cells. and optionally at least one metabolite; and

providing on the extra-cellular side of said semi-permeable substrate at least one metabolite required for proliferation of said cells.

The method of the invention may be used to culture one type of cell. Alternatively. the method may be used to culture two or more types of cells.

The cells may be mammalian cells, although the method of the present invention may be used to expand other types of cells. Examples of mammalian cells that may be expanded using the method of the invention include haematopoietic cells, T cells, B cells, dendritic cells, liver cells,

pancreatic islet cells or genetically modified cells such as chinese hamster ovary (CHO) cells or hybridomas.

The at least protein required for cell proliferation, differentiation and/or genetic modification may be selected from one or more of the group consisting of growth factors, colony stimulating factors, cytokines, cytokine receptors, chemokines, albumin, transferrin, low density lipoproteins, and gene transfer vectors. Examples of growth factors are IL-1, IL-2, IL3, SCF, IL-6, Flt-3 ligand, insulin, thrombopoietin, erythropoietin, EGF, TGF, PDGF, NGF, GF, FGF, etc. Examples of colony stimulating factors are GCSF and GMCSF. Examples of chemokines are MIP1α and insulin-like growth factor. Examples of gene transfer vectors are non-replicative retroviral and adeno-associated viral vectors, lipoplexes and phage vectors. Preferably the molecule or multimolecular complex is present in excess so that it is not significantly depleted by cell consumption or biodegradation. This will vary depending on the specific molecule and the type of cells in culture. The albumin may be present in concentration in the range of about 10 to 50 mg/ml, preferably about 10 mg/ml.

The at least one metabolite required for proliferation may be selected from any anabolite that may be consumed by the cells, for example, glucose, amino acids, vitamins, steroid hormones or any other molecule, except proteins or other larger molecules, required for cell proliferation, differentiation or genetic modification.

The semi-permeable substrate is preferably impermeable to molecules having a molecular weight greater than or equal to about 8,000 and most preferably, greater than or equal to 10,000.

In a particularly preferred embodiment of the first aspect of the present invention, the semi-permeable substrate is cellulose and in the form of a hollow fibre.

Preferably the hollow fibres act as capillaries. Preferably the hollow fibres have radius in the range of about 100 to 400 microns and a wall

thickness in the range of about 6 to 50 μm. A wall thickness of about 7μm is particularly preferred. By use of such semi-permeable hollow fibres, it is possible to maintain glucose, lactate and other metabolites within physiological range by perfusion of media containing these low molecular weight substrates on the extra-capillary side (assuming cells are captured inside fibres). It is not necessary to supplement extra-capillary media with the same proteins required for cell growth resulting in substantial reduction in the amount consumed. Furthermore, because the consumption of these proteins is low, it is not always necessary to perfuse the inside of fibres with media.

Other types of hollow fibres may also be used including ultrafiltration hollow fibre membranes made of cellulose acetate or polysulfone. When such ultrafiltration membranes are used, it will typically be necessary to use the membranes under conditions such that the permeability of the membrane is reduced below its normal level.

Since it is possible that peptides having a molecular weight below 10,000 (eg insulin) will cross the membrane, it may be necessary to include that molecule in the extra-cellular media. It may also be necessary to equalise the osmotic pressure caused by molecules greater than 10,000 molecular weight to prevent influx of water across the semi-permeable substrate into the cellular media. This can be achieved by appropriate selection of the pressure on the extra-cellular side. Alternatively the osmotic pressure can be equalised by including in the extra-cellular media, a molecule that does not cross the cellulose membrane. We have found that molecules significantly less expensive than the proteins used for cell proliferation and growth in the intra-cellular media can be used for this purpose. For example cheaper molecules such as serum albumin (BSA) or dextran (therapeutic grade, molecular weight 70,000) may be included in the extra-cellular media to equalise the osmotic pressure across the semi-permeable substrate. The use of pressure or a molecule such as BSA or

dextran on the extra-cellular side of the substrate provides a process that is significantly less expensive than current tissue culture techniques.

Preferably the cells immobilised on one side of the semi-permeable membrane. Preferably the cells are bound to the semi-permeable substrate by one or more ligands. The ligand may be selected from the group consisting of an antibody, lectin, growth factor and receptor. More preferably, the ligand is an antibody and still more preferably, the ligand is a monoclonal antibody.

The method of the first aspect of the invention may include a preliminary step of separating a desired cell type from sample containing cells of more that one cell type. The separation step may be achieved by immobilising the desired cell type on a semi-permeable substrate and treating the semi-permeable substrate such that the cells not bound to the substrate are removed. The preliminary separation step may be carried out in a separate apparatus, for example the cell separation apparatus described in US Patent No. 5,763,194. Most preferably, the separation step is carried out in the same apparatus as that used to carry out the cell culturing method of the first aspect of the present invention.

In a second aspect, the present invention provides an apparatus for the proliferation and growth of cells including a semi-permeable membrane wherein said semi-permeable substrate is permeable to metabolites but is substantially impermeable to at least one protein required for proliferation, differentiation and/or genetic modification of said cells and is in the form of an array of hollow fibres.

Preferably the device of the second aspect is also capable of separation of specific cells.

The semi-permeable substrate is preferably impermeable to molecules having a molecular weight greater than or equal to about 8,000 and most preferably, greater than or equal to 10,000.

In a particularly preferred embodiment of the second aspect of the present invention, the hollow fibres are cellulose hollow fibres. As

mentioned above, other types of hollow fibres may also be used including ultrafiltration hollow fibre membranes made of polysulfone.

In a further preferred embodiment of the second aspect of the invention, the hollow fibres are located within a housing for containing nutrient media to produce a hollow fibre module. The housing may be cylindrical. The housing may have an inlet and outlet port through which extra-capillary nutrient media may be introduced and withdrawn. The housing may include an inlet and outlet port through which intra-capillary media may be introduced and withdrawn. The hollow fibre module may be of a standard dialyser configuration.

Preferably the housing is in fluid communication with supply means for supplying extra-capillary nutrition media and supply means for suppling intra-capillary culture media. The fluid flow to the housing module may be achieved by use of pump means.

The apparatus of the invention may include one or more gas and heat exchangers through which the media, particularly the extra-cellular media, flows.

The apparatus of the second aspect of the invention may include means for controlling environmental variable(s) for media contained in the supply means, for example, temperature and CO_2 concentration.

Preferably the various operations of the apparatus of the invention are under computer control.

In a particularly preferred form of the apparatus of the invention, the hollow fibres are provided internally with a ligand reactive to the cells Examples of suitable ligands for use in the apparatus of the invention are described above.

The apparatus of the invention is easily scaled-up. For example two or more hollow fibre modules can be used to provide the requisite number of cells during, for example, cell therapy.

The invention will now be described with reference to the following non-limiting embodiments.

EXAMPLES

APPARATUS

An embodiment of an apparatus (bioreactor) of the invention is shown in Figure 1. The bioreactor is designed for combined cell selection and expansion. The necessary components for cell loading and harvesting, or perfusion cultures are shown in boxes A and B, respectively. An autoclavable hollow fibre bioreactor module **10** is housed inside a purpose-built incubator (42x40x47 cm) that controls environmental variables for high-density, perfusion culture (media perfusion rate, temperature & CO_2) in addition to cell selection processes. The incubation chamber in this case maintains temperature at 37°C and CO_2 at 5%.

Hollow fibres are housed within a cylindrical shell of the module using the standard kidney dialyser configuration. The bioreactor module housing **10** has inlet ports **2** and **3** for introduction of intra-capillary media and cells respectively and outlet port **4** for removal of intra-capillary waste. The housing also has inlet and outlet ports **6** and **8** for introduction of extra-capillary media and extra-capillary waste respectively. The device includes a gas and heat exchanger **40** through which passes the extra-capillary media.

Intra-capillary media and waste are stored in containers **32** and **34** respectively. Extra-capillary media and waste are stored in containers **36** and **38** respectively.

Flow control in the device is achieved by stepper motor driven syringe pumps **22** and a peristaltic pump **24** interfaced with an IBM compatible PC (not shown). Fluid paths through the unit are controlled by solenoid pinch valves **26** also interfaced with the IBM compatible PC. The module is rotated by a stepper motor driven turntable (not shown).

The system is controlled using Labview ™ software running on the IBM compatible PC. This software platform provides the tools to create a "virtual instrument" interface which monitors and controls all aspects of devices operation (pump speeds, flow paths, reservoir volumes, temperature and CO_2). The "graphical user interface" (see Figure 2) is used to program and automate the cell separation and culture process.

The system is easily scalable to process from 10^8 to 10^{10} cells using already established cellulose renal dialyser technology. These devices are approved for extra-corporeal use (direct contact with blood), which greatly simplify regulatory approval. Figure 3 is photograph of a medium-scale (10^8 cells / 200 cm^2) and large-scale cellulose hollow fibre module (10^{10} cells / m^2).

An advantage of this embodiment of the apparatus of the present invention is the integration of cell separation and culture as a single platform and growth of cells in the intra-capillary space. Because cells are processed within a closed sterile environment and cell handling is automated, the device is ideally suited for processing of cells under GMP. Other advantages include the ease of scale-up using the compact configuration of a hollow fibre dialyser (1-2 x 10^{10} cells per module) as well as potential cost savings associated with reduced consumption of growth factors and albumin (see below).

In use, the hollow fibres are internally coated with an antibody. For example, to expand $CD34^+$ cells, the hollow fibres are internally coated with anti-CD34 moAb . After capture of $CD34^+$ cells from mononuclear cell concentrates, the cells are expanded inside hollow fibres by perfusion of the extra-capillary space with tissue culture media.

Cells are injected into the module via port 3 by drawing fluid from port 4 followed by injection of fluid by port 2 to wash remaining cells from the header of the module into hollow fibres. Ports 2,3 and 4 are sealed by closure of valves leading to these ports, and the module rotated for 30 minutes whilst

cells attach to hollow fibres. Unbound cells are washed out of the module 10 at low shear stress (10-25 dynes/cm^2) into reservoir 32.

Cells are cultured for 1 to 2 weeks by pumping media (pump 24) through the module via the extra-capillary circuit (ports 6 and 8). The flow rate is approximately 1ml/10^6 cells/day. Media is replaced at regular intervals by pumping media from reservoir 36 into reservoir 38, and replenishing the media from the media refill reservoir. During the culture period it may be necessary to inject intra-capillary media from reservior 32 at a much slower rate (1ml/10^8 cells/day).

At the conclusion of culture cells are harvested by injecting media at a rate which will displace cells out of hollow fibres (>10 dynes/cm^2) into collection bags (reservoir 32 or cell bag). Enzymes, EDTA or other cell releasing agents may be required to detach cells which are still bound.

The device described above has been tested using mobilised peripheral blood and has similar or superior performance to other CD34$^+$ cell selection technologies (Enrichment 1200-fold, Yield 61%).

HIGH-DENSITY CELL CULTURE USING CELLULOSE HOLLOW FIBRES

High-density bioreactors provide a technology for production of mammalian cells or their products using a compact configuration. Another potential benefit of high-density culture is the reduced consumption of expensive or scarce media components such as human albumin, retroviral supernatant or growth factors.

The following experiments establish the feasibility of high-density culture of haematopoietic cells using cellulose hollow fibres.

Aims

1. To establish the final concentration cells will reach when grown inside cellulose hollow fibres given an excess of media in the extra-capillary space

2. To establish which factors limit the growth of cord blood $CD34^+$ cells inside cellulose hollow fibres.
3. To minimise the consumption of expensive components (growth factors and albumin) using the cellulose hollow fibre culture system.
4. Determine optimal extra-and intra-capillary media composition for expansion of cord blood $CD34^+$ cells using the device.

METHODS

Single fibre modules

Single fibre modules were developed to optimise culture conditions for growth of cord blood $CD34^+$ cells. These consist of a single hollow fibre, which is housed in the bottom of a polystyrene tissue culture dish (Figure 4). The ends of the fibre are glued to silastic inlet and outlet tubes so that the inside of fibres can be inoculated with cells. Modules were sterilised using ethanol and UV light irradiation Single fibre modules were inoculated with thawed $1\text{-}2x10^6$ cord blood $CD34^+$ cells per ml. These cells were enriched from cord blood donations (Dr Marcus Vowels, Australian Cord Blood Bank) using MACS kits (Becton Dickinson, Australia), and cryopreserved before thawing.

After inoculation of the single fibre, silastic tubes were clamped using brass clips (Figure 5). The bottom of the tissue culture dish was filled with extra-capillary media. The internal volume of the fibre is only 1.3 μL (π x $100\mu m^2$ x 4cm) whereas the volume of the extra-capillary media is 2 ml. Therefore the extra-capillary media is in excess (~1500: 1). The intra-capillary cell concentration was calculated as follows:

$$\frac{\text{Cell number}}{\text{Volume of fibre segment}}$$

where Volume of fibre segment = $\pi r^2 l$

where r = 100 microns and l is the length of the fibre segment which is counted.

Image analysis and cell counting

The growth of cells inside the hollow fibres could be directly observed using an inverted microscope.

Cells were counted by digital capture (Pulnix CCD camera, TM 1001) and image analysis software (Wit 5. 1). Images of cells growing inside fibres and cell counts were taken on days 0, 2, 5 and 8 of culture. The cell concentration was calculated from the average of 4 images taken at different positions along the fibre.

Extra- and intra-capillary media

Tables 1 and 2 show the constituents of the intra- and extra-capillary media. Cells were cultured in serum-free media (StemPro media and nutrient supplement, Gibco) containing growth factors (IL-3, SCF, TPO and Flt-3 ligand @ either 20 or 100 ng/ml). Some of the constituents of intra- and extra-capillary media were varied, depending on the aim of the experiment.

Table 1. Intra-capillary media

Additive	**Concentration**	**Dilution**	**[Final]**
2-mecaptoethanol	0. 1M	1:100	1mM
Sodium pyruvate	100mM	1:100	1mM
Kanomycin	69 mg/ml	1.5:1000	100 μg/ml
penicillin	41 mg/ml	1.5:1000	62 μg/ml
Growth factors (IL-3, SCF, Flt-3 ligand, TPO)	-	-	20 or 100 ng/ml
StemPro-34 nutrient supplement	N/A	2.6:100	N/A

Table 2. Extra-capillary media

Additive	Concentration	Dilution	[Final]
2-mecaptoethanol	0. 1M	1:100	1 mM
Sodium pyruvate	100mM	1:100	1 mM
Kanomycin	69 mg/ml	1.5:1000	100 μg/ml
penicillin	41 mg/ml	1:5:1000	62 μg/ml
+/-Insulin	-	-	10 μg/ml
+/-Growth factors (IL-3, SCF, Flt-3 ligand, TPO)			20 ng/ml
BSA or Dextran 70 or StemPro-34 nutrient supplement	-	-	10 mg/ml

In the first experiment we determined the effect of attachment on the growth of cord blood $CD34^+$, cells using poly-Lysine as an attachment factor. Poly-L-lysine (100μg /ml was physically adsorbed onto the lumenal surface of hollow fibre modules by incubation overnight at room temperature. Modules were washed with media the next day before injection of cells.

The second experiment examined the influence of substituting Stem pro media supplement in the extra-capillary media with either bovine serum albumin (BSA) or dextran (therapeutic grade, molecular weight 70,000) at the same osmotic pressure (equivalent to 10mg protein per ml). The relative costs of BSA, Dextran or Stem Pro media is $1.56, $0.89 and $80.00 per 100 mls of media. Dextran may have regulatory advantages since it is approved for human infusion.

The third experiment examined the feasibility of not including growth factors or insulin in the extra-capillary media. The cost of growth factors is approximately $A200 per 100 mls of media.

The final experiment examined the influence of intra-capillary growth factor concentration on growth (20ng/ml versus 100ng/ml).

RESULTS

Influence of cell attachment

Cord blood $CD34^+$ cells attach transiently (1-2 days) when fibres are coated with poly-L-lysine. This results in an initial growth lag at 2 days, however there was no significant difference in the expansion of cord blood cells at day 5 or day 8 (Figure 6).

The distribution of cells along the fibre was more uniform when cells were attached. Unattached cells tended to form clumps along the fibre. If the hollow fibre device is used to pre-enrich cord blood by attachment of $CD34^+$ cells using immobilised antibody, it is likely that the effect on growth will not be significant.

It is important to note that cells grew well beyond the maximal density for tissue culture flask culture. Cells in hollow fibres grew to a density of 20-40 $x10^6$ / ml (Table 3). Cord blood $CD34^+$ cells grown in tissue culture flasks do not grow beyond 2 x 10^6 cells/ml.

Effect of growth factor concentration

Removal of growth factors from the extra-capillary media did not have an adverse effect on the growth of cord blood $CD34^+$ cells. In contrast, the intra-capillary concentration of growth factors had a marked influence on the expansion of cells. Figure 7 shows that a fivefold increase in growth factor concentration (20ng/ml versus 100ng/ml) increased the number of cells by at least a factor of two. At higher cell concentrations there was multi-layer deposition of cells in hollow fibres, resulting in an underestimate of the cell number using image analysis software (Figure 8).

A maximal concentration of $32 \mp 9 \times 10^6$ cells/ml (Table 5) was reached at day 8 using growth factors at 100ng/ml. It is estimated that cell numbers may be as much as twice this value because of multilayer cell deposition.

Influence of Insulin, Dextran or BSA in extra-capillary media

The aim of these experiments was to determine whether cheaper alternatives to Stempro media supplement could be substituted into the extra-capillary media. Fully-defined media such as StemPro contains additives such as human albumin, transferin, insulin, and low density lipoproteins. Since it possible that peptides such as insulin cross the membrane (molecular weight 8,000), it may be necessary to include insulin in the extra-capillary media. It is also necessary to equalise the osmotic pressure caused by molecules greater than 10,000 molecular weight to prevent influx of water into the hollow fibres. This can be achieved using a molecule that does not cross the cellulose membrane (molecular weight >10,000). Alternatively, osmotic pressure may be equalised by appropriate selection of the pressure of the media on the extra-capillary side.

Figure 9 shows the growth curves for this series of experiments. StemPro media supplement was the best extra-capillary media, however BSA on its own could be substituted for Stempro media supplement with only a marginal (not statistically significant) decrease in growth. Surprisingly insulin in the extra-capillary media appeared to reduce cell growth ($p<0.05$). There was poor growth and cell necrosis when dextran was substituted in the extra-capillary media.

An important function of albumin is to bind calcium and small molecules such as insulin. Dextran does not bind these molecules, and it may be necessary to adjust calcium so that intra-capillary free calcium levels are in the physiological range.

CONCLUSIONS

The small-scale single fibre studies have established proof of principle of high-density haematopoietic cell culture using cellulose hollow fibres. An optimised system should support at least 50 million cells/ml. This will

reduce the size of current flask and bag tissue culture systems by a factor of at least 50.

It is also feasible to exclude growth factors from the extra-capillary media, and substitute Stem Pro media supplement with a simpler and cheaper alternative such as bovine serum albumin (dialysed) or dextran or by use of pressure.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this day of September 1999

Unisearch Limited
Patent Attorneys for the Applicant:

F B RICE & CO

Figure 1 Cell manipulation system (CMS)

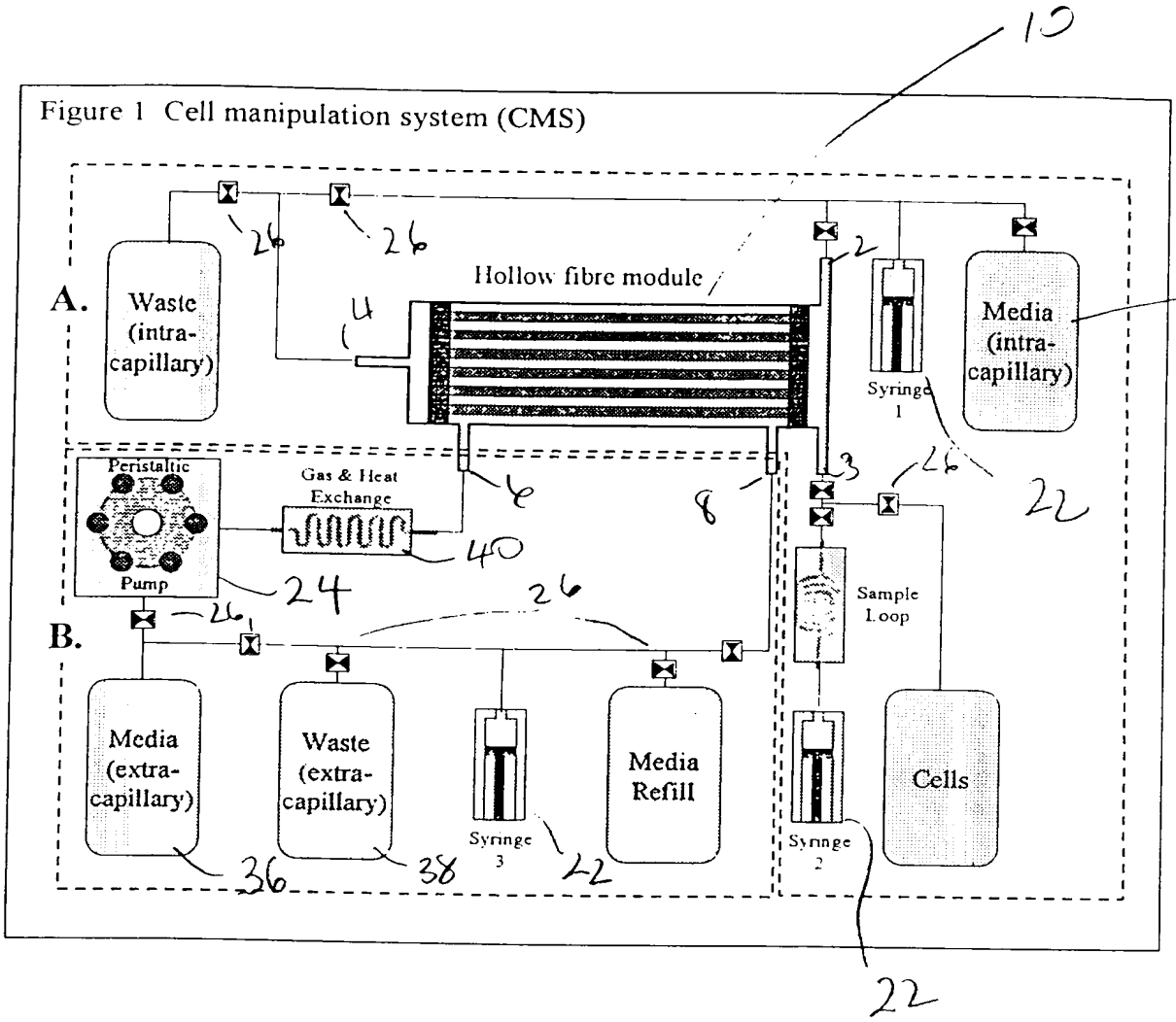


Figure 3 Hollow fibre modules (200 cm^2, 1 m^2)

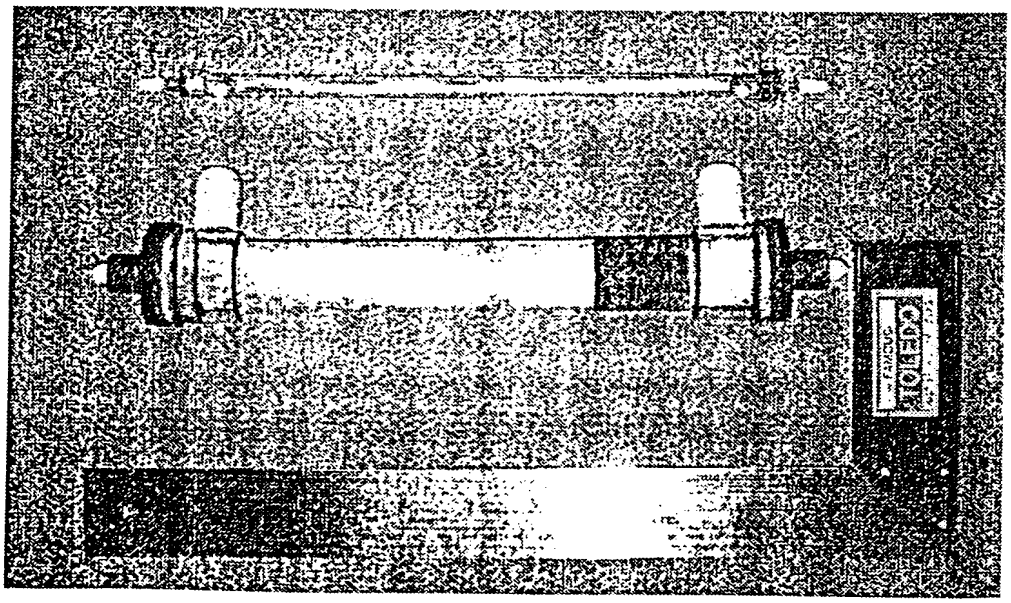


Figure 2 Graphical User Interface

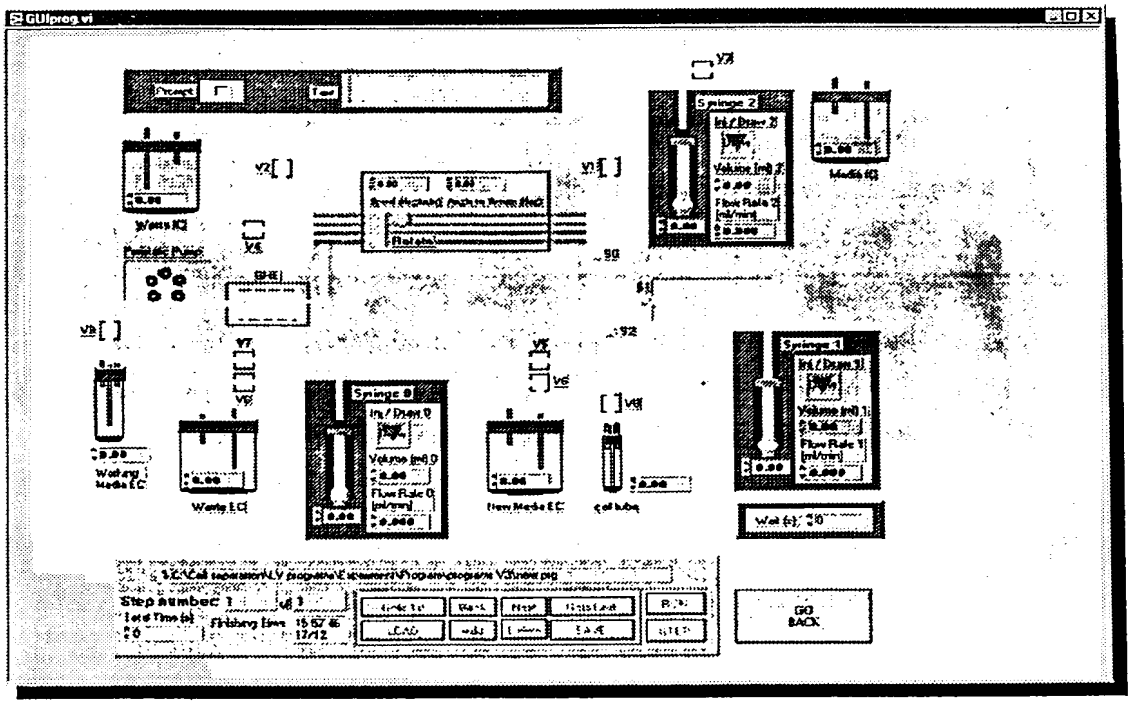


Figure 4 Single fibre module (empty)

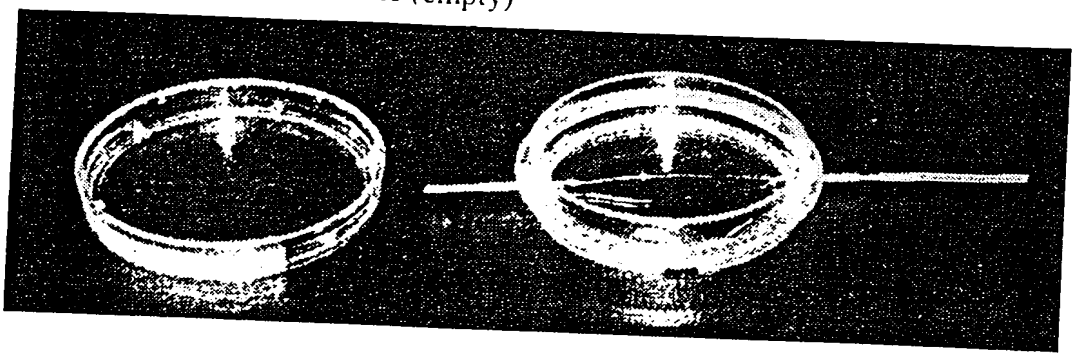


Figure 5. Single fibre module filled with culture media.

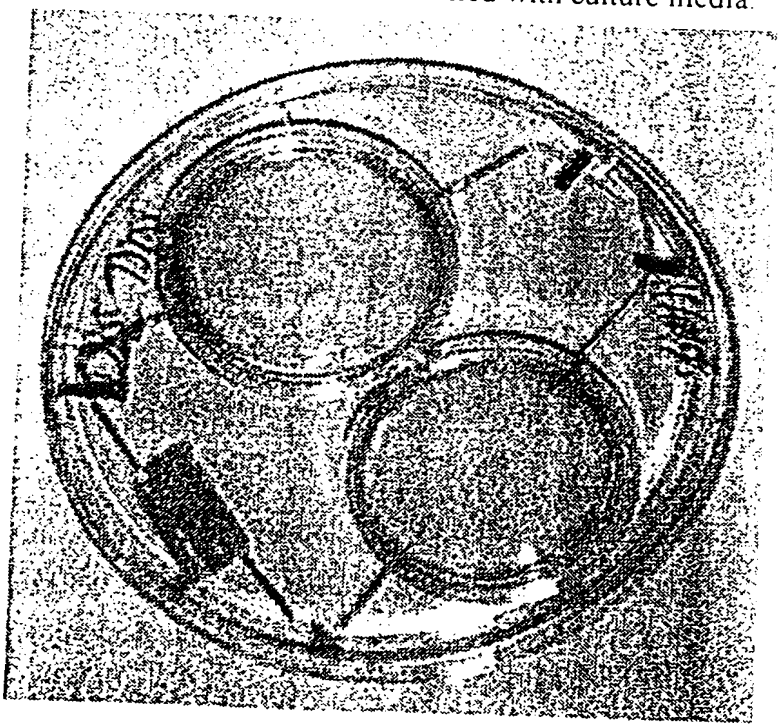


Figure 6. Effect of cell attachment on growth of cord blood $CD34^+$ cells (mean±SEM)

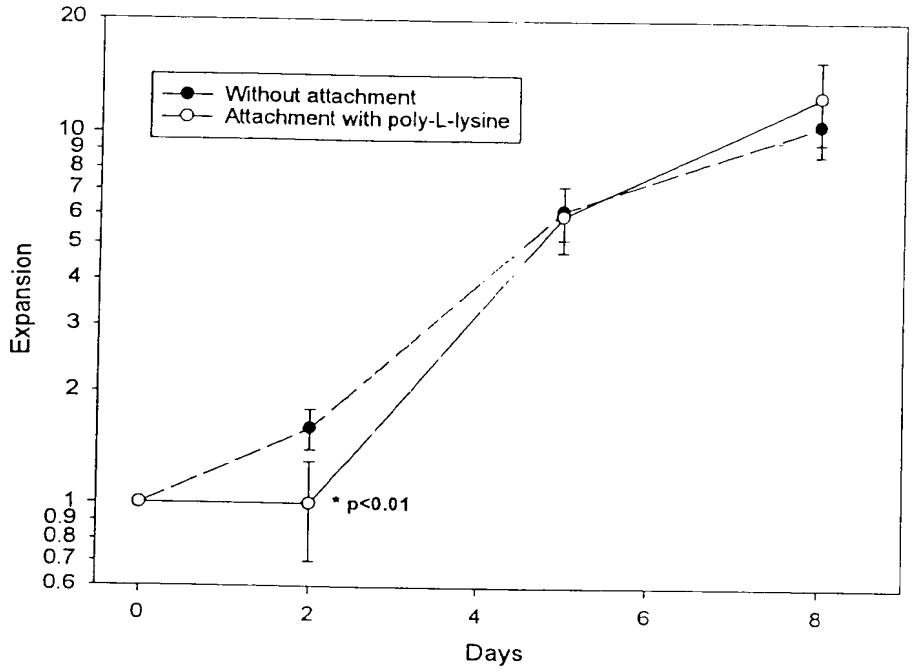
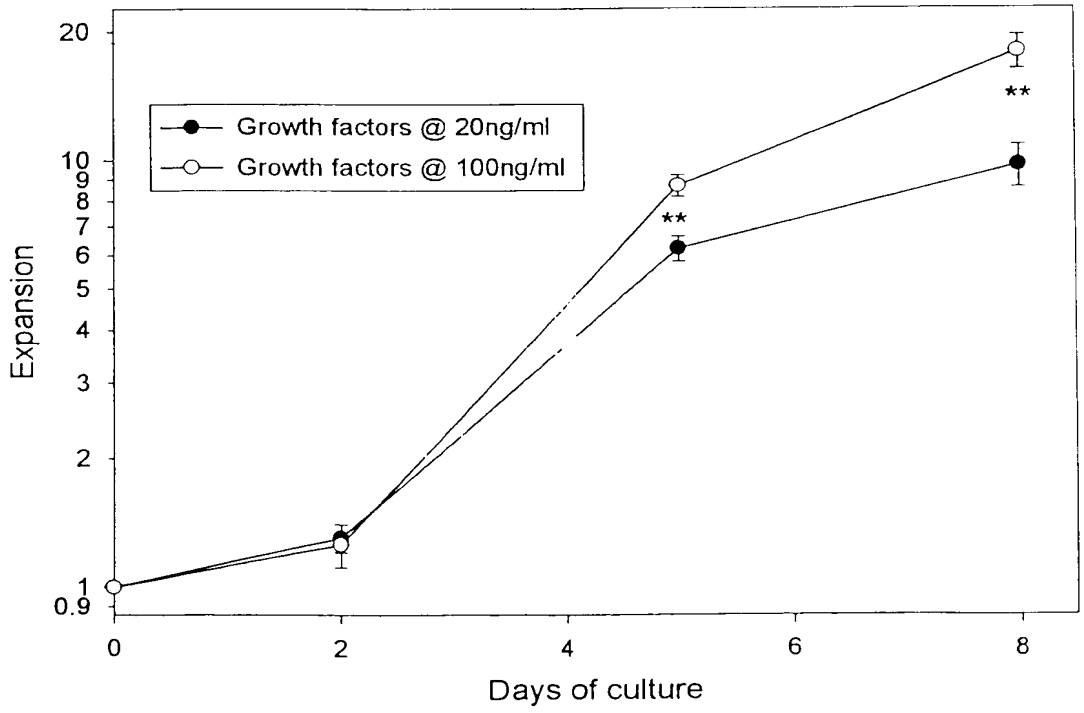


Figure 7. Effect of intracapillary growth factor concentration (mean±SEM) ** p<0.005



8. Photomicrographs of single hollow fibres. Effect of intracapillary growth factor ntration

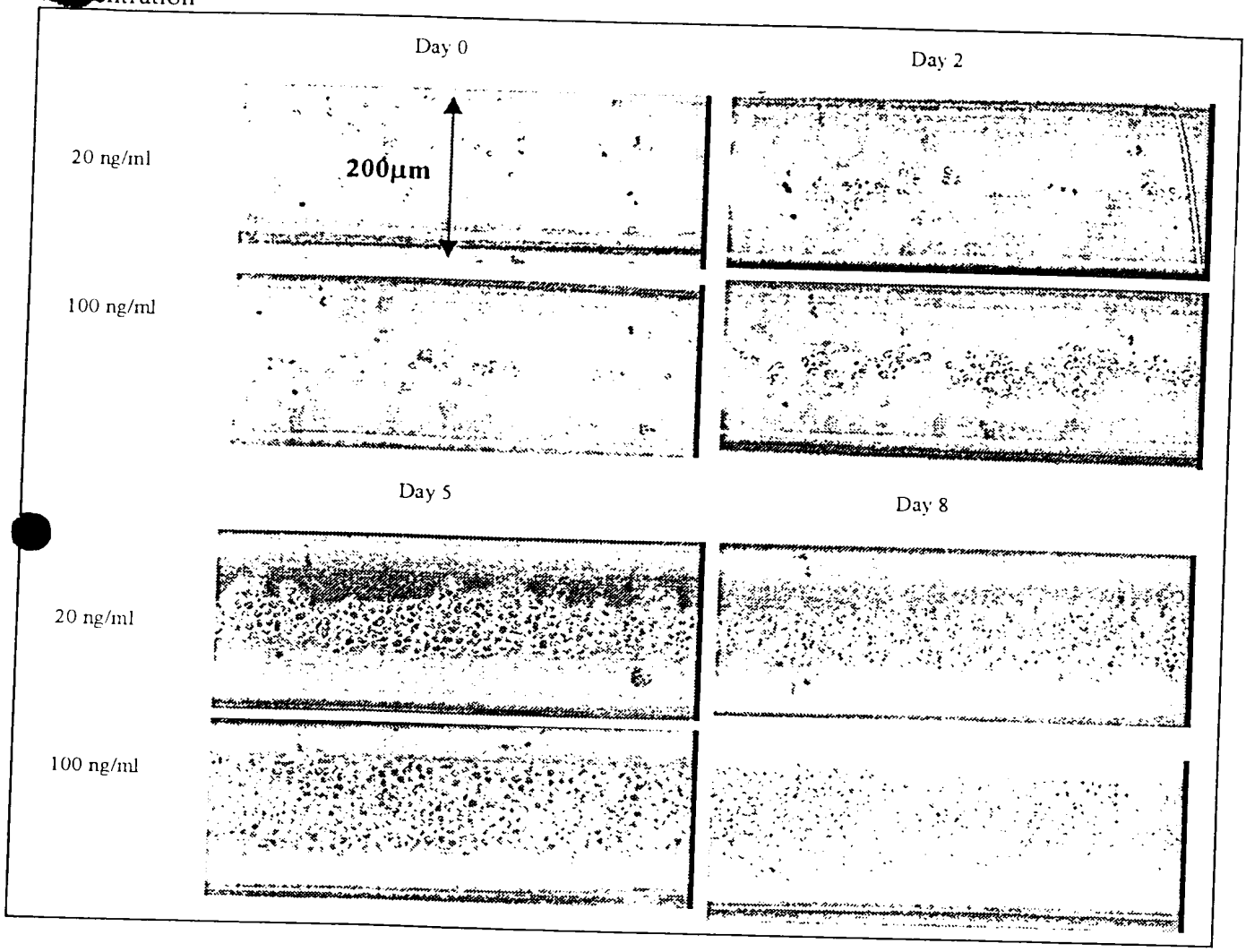


Figure 9 Influence of insulin, BSA and dextran in extra-capillary media (mean±SEM) * $p<0.05$, ** $p<0.01$